Journal of Medical Virology 53:127–138 (1997)

Differentiation of Core Gene Products of the Hepatitis B Virus in Infected Liver Tissue Using Monoclonal Antibodies

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Hepatitis B virus (HBV) core gene translational products were localised previously in the cytoplasm and/or in the nuclei of infected cells. We investigated in naturally infected human hepatocytes whether this variation in the subcellular expression is due to differences in the presence of assembled core particles and other core gene derived proteins, the expression of HBeAg and the processing of liver tissue. By immunostaining of liver specimens infected with HBeAg-positive and HBeAg-minus variants of HBV, using monoclonal antibodies specific for assembled core particles and for various epitopes on denatured core protein, it was shown that virtually all immunoreactive core gene products are assembled into core particles. The latter are present both in the nuclei and in the cytoplasm of hepatocytes, independent of the infecting virus strain. A marked reduction or absence of immunoreactivity, observed with some monoclonal antibodies, was shown to result from nucleotide sequence variations within or close to the corresponding epitope. These results demonstrate that immunoreactive products, derived from the HBV core gene, in the nuclei and cytoplasm of human hepatocytes represent assembled core particles and that monoclonal antibodies with known recognition sites can reveal region-specific core gene variation of the infecting HBV population. ***J. Med. Virol. 53:127–138, 1997.***

KEY WORDS: hepatitis B virus; hepatitis B core antigen; monoclonal anti-HBc; chronic hepatitis B

INTRODUCTION

The hepatitis B virus (HBV) core gene (C-gene) is divided into a preC- and a C-region by two translational start codons. Initiation at the first AUG (preC-AUG) leads to the synthesis of P25 protein, which undergoes two processing steps [Jean-Jean et al., 1989]. Cotranslational cleavage of the aminoterminal end of P25 removes the signal sequence and results in P23 protein, a processing intermediate found in transfected cells both in the cytoplasm and in the nucleus [for review see Standring, 1991; Ou et al., 1989]. Most of these molecules undergo a further proteolytic cleavage, at the carboxyterminal end, and are finally secreted as nonparticulate HBeAg. A minor fraction of P25 seems to be processed only at the carboxyterminal end and secreted [Takahashi et al., 1991]. Initiation of translation at the second AUG of the C-gene results in synthesis of the nucleocapsid protein (HBcAg, P21) which assembles into core particles [for review see Standring, 1991]. A number of HBV variants have been identified which can only synthesize HBcAg but no precore-derived products due to various mutations in the preC region [for review see Miska and Will, 1993]. Other HBV variants with point mutations or deletions in the core gene have been described in patients with chronic hepatitis B and these genomic variants predictably express significantly altered HBc proteins [Miska and Will, 1993; Miska et al., 1993; Akarca and Lok, 1995; Ackrill et al., 1993; Marinos et al., 1996]. Taken together, a variety of normal C-gene products and mu-

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Contract grant sponsor: Wilhelm-Sander-Stiftung; Contract grant sponsor: Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie.

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Accepted 9 May 1997

tant proteins are predicted to be expressed from the wildtype and mutant HBV variants, respectively. However, little is known about their cellular localisation in infected liver tissue, as only few well defined monoclonal antibodies which can differentiate between various C-gene products or between assembled and nonassembled core particles are available so far.

For several reasons the intracellular localisation, and trafficking of C-gene products are of particular interest. First, the nucleocapsid has a critical role in the HBV life cycle, as it is required for RNA pregenome encapsidation and DNA synthesis, which take place within the core particles in the cytoplasm (Nassal and Schaller, 1993). Second, C-gene products are major targets of the host immune response, particularly in chronic HBV infection [Chisari and Ferrari, 1995]. Third, the pattern of the intracellular distribution of C-gene derived products, as detected by immunostaining, has been associated with different levels of virus replication, liver disease activity [Hsu et al., 1987, Naoumov et al., 1990] and liver cell regeneration (Guidotti et al., 1994). Fourth, intracellular accumulation of nonenveloped core particles can be cytopathic [Roingeard et al., 1990]. Fifth, the presence of mutant core protein in liver cells can interfere with the efficiency of virus production [Scaglioni et al., 1994] and possibly with response to interferon therapy [Naoumov et al., 1995].

The large number of C-gene derived products, overlapping in sequence and partially also in structure, as well as the lack of well defined antibodies, had made it difficult to localise each of them individually in the cellular compartments. In liver specimens from HBV infected patients core particles have been detected by electron microscopy both in hepatocyte nuclei and cytoplasm [Gudat et al., 1975]. In contrast, nucleocapsid particles expressed in frog oocytes and in nonregenerating hepatocytes of transgenic mice are localised almost exclusively either in the cytoplasm or in the nuclei, respectively (Zhou and Standring, 1991; Guidotti et al., 1994). From these and additional studies it is not clear whether there are nonassembled and aberrant C-gene products in infected human hepatocytes, apart from assembled core particles.

Recently, a panel of monoclonal antibodies which recognises exclusively assembled core particles or defined sequences of nonassembled C-gene products in denatured form have been described [Bichko et al., 1993; Apsalons and Bichko, 1994; Sällberg et al., 1993]. This provides the opportunity to analyse the presence and localisation of C-gene derived products in naturally infected human livers. Using these monoclonals and a polyclonal antibody against hepatitis B core protein we demonstrate that most of the immunoreactive C-gene products in HBV infected liver tissue are present as assembled core particles which are partially denatured upon formalin fixation and that monoclonal antibodies with known recognition sites on the denatured core protein can detect the presence of site-specific C-gene variation of the infecting HBV strain.

MATERIALS AND METHODS

Patients Details

The study population includes two groups of patients (Table I). Group A comprises 15 patients with chronic HBV infection, seropositive for hepatitis B surface antigen (HBsAg) for more than 12 months. A percutaneous liver biopsy was performed in each patient as part of their diagnostic evaluation and C-gene products were analysed both in cryostat, as well as in formalin-fixed, paraffin-embedded sections. All 15 patients were with ongoing HBV replication (seropositive for HBV DNA) of whom 12 were HBeAg positive and three were anti-HBe positive. Liver histology in 12 HBeAg positive patients showed chronic hepatitis with minimal to mild hepatic inflammation in seven and chronic hepatitis with moderate to severe inflammatory activity in five. Liver histology was assessed on formalin fixed sections, according to the recent recommendations for classification of chronic hepatitis [Desmet et al., 1994]. None of the 12 HBeAg positive patients had received any treatment prior to this study. They were all seronegative for the human immunodeficiency virus, hepatitis Delta virus (HDV) and hepatitis C virus (HCV). The three patients with anti-HBe and HBV-DNA had received orthotopic liver transplant for HBsAg positive liver cirrhosis and developed subsequently recurrent HBV infection in the liver graft. The liver specimens used in this study were obtained between 12 to 20 months after transplantation. Liver graft histology showed chronic hepatitis with moderate inflammatory activity in two and cirrhosis with moderate activity in one. One of the liver graft recipients was co-infected with HDV and showed delta antigen in the liver tissue, while the remaining two transplant recipients were HDV and HCV negative.

Group B comprised eight patients from whom only formalin-fixed, paraffin-embedded liver sections were available (Table I). Five of these patients were untreated chronic HBV carriers, seropositive for anti-HBe and HBV-DNA. Liver histology showed chronic hepatitis with severe inflammatory activity and moderate fibrosis in three, and liver cirrhosis with severe activity in two patients. The remaining three patients had received an orthotopic liver transplant and subsequently developed HBV reinfection in the liver graft. All three were seropositive for HBeAg and HBV-DNA at the time of the study and the liver biopsies were performed between 12 and 40 months after liver transplantation. They showed chronic hepatitis with mild activity in two and cirrhosis with moderate inflammatory activity in one patient. At the time of liver biopsy the immunosuppression in the transplant patients studied was as follows—two patients were on triple therapy with cyclosporine, azathioprine, and 5 mg prednisolone; one was receiving cyclosporine and 5 mg prednisolone, and the remaining three liver graft recipients were receiving cyclosporine and azathioprine.

TABLE I. Characteristics of Patients With Chronic HBV Infection Studied*

Group	Patient no.	Sex	Serum HBeAg/anti-HBe	Serum HBV-DNA pg/ml	Serum AST IU/I	Liver histology
A	1	F	+/−	243	22	CH with minimal to mild activity
	2	M	+/−	329	41	-″- -″-
	3	M	+/−	1,045	40	-″- -″-
	4	M	+/−	71	35	-″- -″-
	5	M	+/−	574	41	-″- -″-
	6	M	+/−	26	56	-″- -″-
	7	M	+/−	16	34	-″- -″-
	8	M	+/−	120	356	CH with moderate to severe activity
	9	M	+/−	159	60	-″- -″-
	10	M	+/−	210	132	-″- -″-
	11	M	+/−	775	81	-″- -″-
	12	M	+/−	5	160	-″- -″-
	13	M	−/+	1,845	120	OLT, CH with moderate activity
	14	M	−/+	484	212	OLT, Ci with moderate activity
	15	M	−/+	92	400	OLT, CH with moderate activity
B	16	M	+/−	840	25	OLT, CH with mild activity
	17	M	+/−	810	31	OLT, CH with mild activity
	18	F	+/−	138	61	OLT, Ci with moderate activity
	19	M	−/+	1200	521	Ci with severe activity
	20	F	−/+	420	288	CH with severe activity
	21	F	−/+	380	420	-″- -″-
	22	M	−/+	80	180	Ci with severe activity
	23	M	−/+	200	210	CH with severe activity

*OLT, Orthotopic liver transplant recipient with a recurrent HBV infection in the graft; CH, chronic hepatitis; Ci, liver cirrhosis; -″- -″-, indicates the liver histology is the same as above.

Detection of Core Protein in Cryostat Liver Sections

Liver specimens from patients in Group A were divided into two parts. One was fixed in 10% buffered formaldehyde for 18 to 24 hours and embedded in paraffin wax. Another part was snap frozen in liquid nitrogen-cooled isopentane and stored at −70°C. From these stored samples, 5 μm cryostat sections were cut and mounted on slides coated with 0.1% poly-L-lysine and air dried. For immunostaining the antibodies were applied to both cryostat and formalin-fixed liver sections. In preliminary experiments we have established that there is no difference in the detection of particulate core protein in untreated cryostat liver sections or after 5 min fixation with cold acetone. As the latter approach improved liver tissue morphology, this was maintained standard for the entire study without any other pretreatment of the cryostat sections before applying the corresponding antibodies.

For immunostaining cryostat sections were rehydrated and incubated with the corresponding antibodies for 60 min at 37°C. After the incubation with the primary antibodies, the endogenous peroxidase activity in the liver sections was blocked with methanol-1% H_2O_2 for 15 min at room temperature. Rabbit anti-mouse and swine anti-rabbit antibodies, both labelled with peroxidase (DAKO Ltd., High Wycombe, UK) were consecutively applied for 30 min each. The peroxidase-reaction was developed with diaminobenzidine as a substrate. For comparison, polyclonal rabbit anti-HBc (DAKO Ltd.) was applied and detected by peroxidase-labelled swine anti-rabbit antibody and rabbit peroxidase anti-peroxidase (PAP) complexes (DAKO Ltd.).

Detection of Core Protein in Formalin-Fixed, Paraffin-Embedded Liver Sections

The expression of core protein was detected using avidin-biotin immunoperoxidase technique, as described previously [Naoumov et al., 1990]. Briefly, the sections were deparaffinised, washed with alcohol, treated with methanol-1% H_2O_2, and rehydrated. Mouse monoclonal antibodies or the polyclonal rabbit antibody were added and subsequently detected using biotinylated rabbit anti-mouse antibody or biotinylated goat anti-rabbit antibody respectively, and streptavidin ABC peroxidase (all reagents from DAKO Ltd.).

A semiquantitative assessment of the immunoreactivity in cryostat and in formalin-fixed sections was carried out independently by two observers by scoring the proportion of positive cells in four microscopic fields at magnification ×250. The total score for each antibody was stratified as 0%; 1% in case of single positive hepatocytes; 10% for small groups of positive hepatocytes; 30%; 50%; or 80% of hepatocytes showing positive immunostaining.

As specificity controls, cryostat and paraffin sections of liver specimens from five patients with primary biliary cirrhosis, seronegative for all HBV markers and a normal cut-down liver were used and tested with all monoclonal antibodies and with the polyclonal rabbit antibody to hepatitis B core protein. In addition, the specificity of the positive immunostaining in hepatitis B specimens was verified by replacing the primary

TABLE II. Specificity of Monoclonal Antibodies to Hepatitis B Core Protein*

No.	MAb	Immunogen	Ig class	Epitope
1	C 3-1	native HBc	IgG3	conformational only
2	C 1-5	native HBc	IgG2a	aa 74–89 conformational plus linear
3	11E2	denatured HBc	IgG1	aa 2–10
4	13A9	-″-	IgG2b	aa 134–140
5	13C9	-″-	IgG2a	aa 134–140
6	13D3	-″-	IgM	aa 143–146
7	13F12	-″-	IgM	aa 146–154

*aa, amino acid positions of the epitope, numbered from the beginning of HBc protein, according to Galibert et al. [1979]; ″, indicates the same as above.

monoclonal or polyclonal antibody by buffer only and by monoclonal anti-rubella antibody or normal rabbit serum, respectively. No positive reaction was observed in any of these experiments.

Antibodies

The mouse monoclonal antibodies (MAbs) specific for native, conformational epitopes of assembled core particles and for various epitopes of denatured C-gene products (Table II) were described previously [Bichko et al., 1993; Sällberg et al., 1993; Apsalons and Bichko, 1994]. Only one of the monoclonal antibodies used (MAb 11E2) reacts with HBeAg. The fine specificity of all monoclonal antibodies was mapped in an ELISA assay with overlapping core peptides and fusion proteins, in competition studies using native HBeAg, core particles, peptides or fusion proteins, and by immunoblotting. These monoclonal antibodies can be obtained from Chemicon International Ltd. (Harrow, UK). The polyclonal rabbit antibody used was a commercially available one (DAKO Ltd.) which is frequently applied in routine clinical immunohistology.

HBV-DNA Sequencing Analysis

The HBV C-gene was amplified from the HBV-DNA in serum samples by polymerase chain reaction and directly sequenced as previously described [Miska et al., 1993]. The serum samples used for this analysis were taken at the time of the liver biopsy from each patient.

Serological Assays

HBsAg, HBeAg, anti-HBe, anti-HDV, and anti-HCV were tested using commercially available kits (Abbott Diagnostics, Maidenhead, UK; Sorin Biomedica, Saluggia v.c., Italy, and UBI HCV EIA, United Biochemical Inc., Lake Success, NY). Serum HBV-DNA was quantitated with the solution hybridisation assay Genostics (Abbott Diagnostics).

RESULTS

Detection of Assembled Core Protein

The monoclonal antibody C3-1 recognizes a conformational epitope present exclusively on assembled core particles and does not react with any other form of C-gene derived products tested so far [Bichko et al., 1993]. The analysis of immunostaining with MAb C3-1 in cryostat sections from all but two patients in group A showed cytoplasmic and/or nuclear staining in a large proportion of hepatocytes (Table III; Fig. 1a). In those cells with both cytoplasmic and nuclear staining the latter was much more intense. This observation indicates that the assembled, nonenveloped core protein is present in the cytoplasm and even in higher amount in the nucleus. When paraffin embedded sections from the same liver specimens were tested with MAb C3-1 no cytoplasmic staining was observed in any of the samples (for an example see Fig. 1b). In addition, five specimens (patients 4, 5, 6, 10, and 15) with exclusive nuclear staining became negative (Table III) and further four specimens (patients 1, 2, 3, and 14; Table III) showed a reduced nuclear immunostaining. Consistent with the specificity of MAb C3-1, these data indicate that most assembled core particles, present in naturally infected human hepatocytes, loose the conformational epitope recognised by MAb C3-1 during formalin fixation and paraffin embedding.

The subcellular localisation of particulate core protein in hepatocyte cytoplasm and nuclei in the seven patients with HBeAg positive chronic hepatitis with minimal to mild activity was similar to that observed in five HBeAg positive patients with moderate to severe hepatitis. In general, the proportion of hepatocytes with core particles was lower in patients with greater hepatic inflammation. As expression of HBeAg was reported to influence HBV replication [Lambert et al., 1994] we also tested whether core protein expression and distribution is different in livers infected with preC mutant HBV, unable to express HBeAg, compared to those infected with the preC wildtype virus. Three of the patients tested with MAb C3-1 were anti-HBe positive (patients 13–15, Table I). Direct sequencing of the HBV-DNA isolated and amplified from the serum of these patients showed in all three a G to A substitution at position 1896 [nomenclature according to Galibert et al., 1979] in the preC region. This mutation creates a stop codon which prevents the expression of all precore derived proteins including HBeAg. Similar to the HBeAg positive patients, the liver specimens from pa-

TABLE III. Detection of Core Gene Products in Cryostat and Paraffin-Embedded Liver Tissue Using Monoclonal Antibodies Recognizing Conformational Epitopes and a Polyclonal Antibody*

Patient no.	C1-5 MAb				C3-1 MAb				PAb anti-HBc			
	Cryostat		Paraffin		Cryostat		Paraffin		Cryostat		Paraffin	
	%	site	%	site	%	site	%	site	%	site	%	site
1	80	N/C	80	N/c	50	N/c	50	N	10	N	50	N
2	80	N/c	80	N/c	50	N/c	50	N	10	N	50	N
3	80	N/C	50	N	80	N/c	30	N	30	N	30	N
4	30	N/c	1	N	30	N	0	—	0	—	50	N/c
5	50	N/c	1	N	10	N	0	—	1	N	80	N/C
6	30	N	0	—	10	N	0	—	0	—	80	N/c
7	0	—	0	—	50	n/C	0	—	0	—	80	n/C
8	0	—	0	—	50	n/C	0	—	0	—	50	n/C
9	1	N	0	—	0	—	0	—	0	—	30	N/c
10	30	N/c	30	N	10	N	0	—	0	—	80	N/c
11	80	N/C	1	N	50	n/C	1	N	0	—	80	n/C
12	0	—	0	—	0	—	0	—	0	—	1	N
13	80	N/C	0	—	50	N/c	0	—	50	N	80	N/c
14	50	N/c	50	N/c	30	N/c	30	N	10	N	50	N/c
15	50	N/C	10	N	30	N	0	—	10	N	80	N/c

*Patients are shown with the same number as in Table I. MAb, Monoclonal antibody; PAb, rabbit polyclonal anti-HBc; N, nuclear; C, cytoplasmic; N/c, predominantly nuclear and weaker cytoplasmic staining; n/C, predominantly cytoplasmic staining with nuclear core protein only in a small number of hepatocytes; N/C, strong core protein staining both in the nuclei and in the cytoplasm.

tients infected with preC mutant HBV showed assembled core particles in nuclei and in the cytoplasm. These results strongly suggest that expression of HBeAg does not influence the subcellular localisation of core particles.

Detection of Assembled and Denatured Core Protein With MAb C1-5

The MAb C1-5 is known to react with an epitope on assembled core particles, but also with denatured HBcAg on immunoblots [Bichko et al., 1993]. However, it does not react with monomers or dimers of core as shown by in vitro studies [V. Bichko, unpublished data]. This provides the opportunity to test to what extend core particles are degraded during formalin fixation and paraffin embedding. The analysis of cryostat sections of all but two patients in Group A showed a staining pattern similar to that seen with MAb C3-1 [Table III; Fig. 1c]. However, the proportion of core positive hepatocytes and those with nuclear and cytoplasmic staining was higher. Consistent with the mapping data, these results indicate that both MAb C3-1 and C1-5 recognise a conformational epitope on assembled core particles also in immunohistochemistry. The quantitative difference could be due to a higher prevalence of the C1-5 epitope on core particles or by the presence of the same epitope on additional immunoreactive C-gene products. As the number of cells showing cytoplasmic staining was reduced in formalin-fixed compared to cryostat sections (Table III; Fig. 1d) we assume that formalin fixation does not induce the same conformational change of the core particles as SDS gel electrophoresis and immunoblotting. Otherwise, the same staining intensity should have been observed in cryostat and paraffin sections with MAb C1-5.

Detection of C-Gene Products With a Polyclonal Rabbit Antibody

The epitopes recognized by the commercially available antiserum used in our study and in routine immunohistochemistry, as carried out in many laboratories, are unknown. However, this antiserum was produced against recombinant core particles and has been shown to recognize both particulate and nonparticulate forms of C-gene products [Stahl et al., 1982]. It was therefore of interest to compare its staining pattern with that of the well defined MAbs C3-1 and C1-5. Immunostaining of cryostat sections with the polyclonal antibody showed in none of the specimens cytoplasmic staining and less frequently and less intense nuclear staining than the two monoclonal antibodies (Table III). The qualitative and quantitative differences of the staining pattern suggest that either the two epitopes on assembled core particles seen by C3-1 and C1-5 are not recognised by this rabbit polyclonal antibody or the corresponding antibodies are of very low titer. The proportion of positive hepatocytes, as detected by immunostaining of paraffin sections from the same patients with the polyclonal antibody, was increased dramatically. This was seen in 11 out of 15 specimens showing both nuclear and cytoplasmic staining and with four specimens exhibiting nuclear staining only (Table III). The dramatic increase in the positive signal in paraffin sections is obviously due to the denaturation of the core particles induced by formalin fixation and paraffin embedding, leading to the exposure of many new epitopes which are recognised by the polyclonal antibody. This is most strikingly evident in the staining of sections from patients 7 and 8, which were negative in the cryostat sections, but strongly positive in those fixed with formalin. The reverse was observed with MAb C3-1. These observations indicate that the epitope recog-

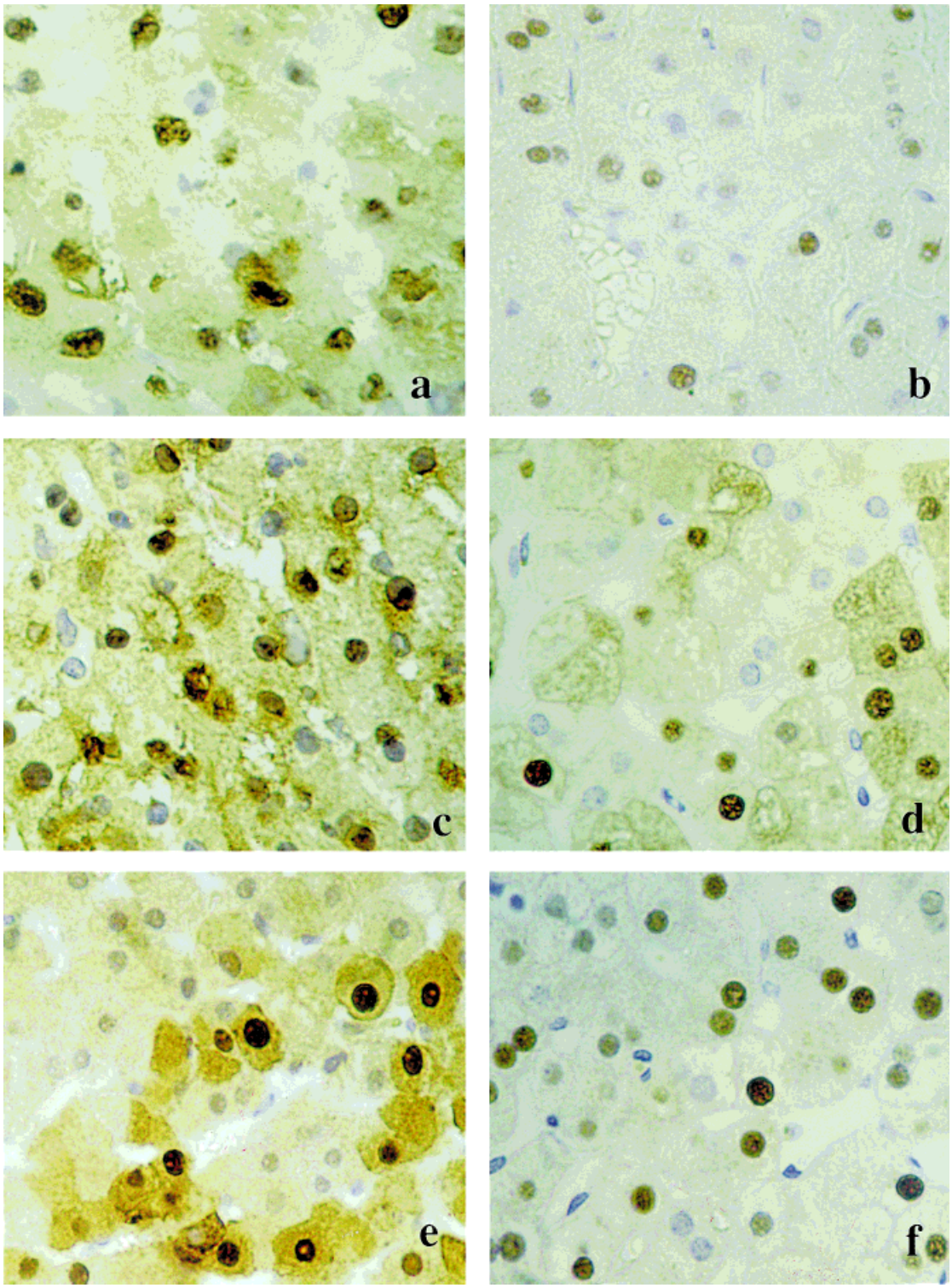

Fig. 1. Immunostaining of cryostat sections (CS) and formalin-fixed, paraffin-embedded sections (FPS) of HBV infected liver specimens with monoclonal and polyclonal antibodies recognising epitopes of particulate and denatured core proteins. **a:** Mab C3-1 on CS from patient No. 3 and **(b)** MAb C3-1 on FPS from the same liver specimen. **c:** MAb C1-5 on CS from patient No. 1 and **(d)** MAb C1-5 on FPS from the same liver specimen. **e:** MAb11E2 on FPS from patient No. 3 and **(f)** polyclonal anti-HBc antibody on FPS from the same specimen. **g:** Predominantly cytoplasmic localization of core protein with the polyclonal anti-HBc on FPS from patient No. 7. **h:** MAb13A9 on FPS from patient No. 1 reveals only nuclear localization of core protein. **i:** Positive immunostaining with MAb13D3 on FPS from a liver specimen infected with HBV genotype D, and **(j)** The same pattern is revealed with the polyclonal anti-HBc (patient No. 14). **k:** Negative immunostaining with Mab13D3 on FPS from a liver specimen infected with HBV genotype A, and **(l)** Positive staining on FPS from the same specimen using polyclonal anti-HBc (patient No. 17).

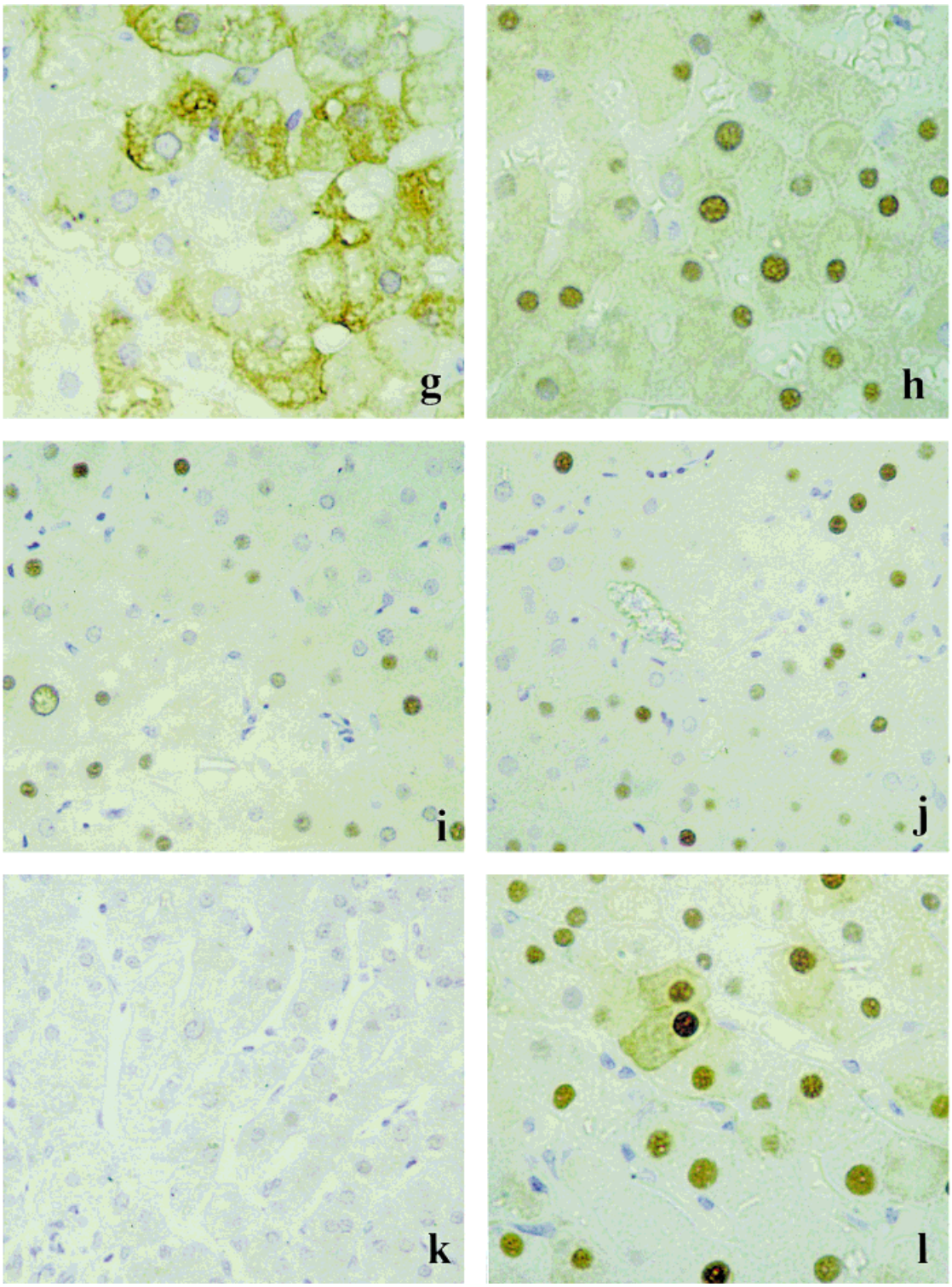

Fig. 1. Continued.

nized by MAb C3-1 in these liver sections is not seen by the polyclonal antiserum. Interestingly, MAb C1-5 did not stain the sections from patients 7 and 8, independent of the type of preparation (Table III), suggesting that the core particles of the HBV strains in these patients differ substantially from those in the other cases (see below). This was also evident from the predominant cytoplasmic and meagre nuclear immunoreactivity of the sections when using the polyclonal antibody (Fig. 1g).

TABLE IV. Detection of Epitopes on Denatured Core Protein in Formalin-Fixed, Paraffin Embedded Liver Tissue*

Patient	Pab anti-HBc		Mab 11E2		Mab 13A9/13C9		Mab 13D3		Mab 13F12	
no.	%	site	%	site	%	site	%	site	%	site
1	50	N	80	N	80	N	10	N	30	N
2	50	N	30	N	80	N	10	N	30	N
3	30	N	80	N/C	50	N	1	N	50	N
4	50	N/c	50	N/c	50	N	1	N	10	N
5	80	N/C	50	N/c	80	N	0	—	30	N
6	80	N/c	30	N	30	N	1	N	30	N
7	80	n/C	0	—	80	n/C	0	—	0	—
8	50	n/C	0	—	30	n/C	0	—	0	—
9	30	N/c	1	N	30	N	0	—	1	N
10	80	N/c	50	N/c	10	N	1	N	10	N
11	80	n/C	80	n/C	80	n/C	1	N	1	N
12	1	N	1	N	1	N	0	—	0	—
13	80	N/c	80	N/C	80	N	80	N/C	30	N
14	50	N/c	80	N/c	50	N	50	N	10	N
15	80	N/c	80	N/C	80	N	50	N	50	N

*aa, Amino acid positions of the epitope, recognised by the corresponding monoclonal antibody (Mab). Numbering is according to Galibert et al. [1979].

Detection of Short, "Linear" Epitopes Using Monoclonal Antibodies

All five monoclonal antibodies, which do not recognise assembled core particles are directed to short HBcAg sequences (Table II) and only react with partially or fully denatured C-gene derived proteins [Bichko et al., 1993; Apsalons and Bichko, 1994]. This provided the opportunity to test whether there is a fraction of nonassembled core protein, in addition to the assembled core in HBV infected hepatocytes. When tested on cryostat sections, none of these MAbs showed a positive signal, indicating that the vast majority of C-gene products in the hepatocytes are assembled core particles. When paraffin sections from the same liver specimens were analysed with these MAbs all showed positive immunostaining (Table IV). This is consistent with the new epitopes that are predictably created as a result of core particle denaturation during the specimen processing. However, there were marked quantitative and qualitative differences in the individual immunostaining pattern. For example, using MAb 11E2 the immunoreactivity in paraffin sections (Table IV) correlated quite well with the pattern of subcellular localization and the proportion of positive hepatocytes as detected with MAb C1-5 on cryostat sections from the same patients (Table III). In some patients, like No. 1, 3, and 14 (Table IV), MAb 11E2 showed much stronger cytoplasmic and/or nuclear immunostaining (Fig. 1e) in comparison to that detected with the polyclonal antiserum (Fig. 1f). In contrast, MAbs directed to carboxyterminal sequences (region aa 134–154, Table II) detected C-gene products almost exclusively in hepatocyte nuclei (Fig. 1h).

Effect of Core Gene Variation on Epitope Detection With Monoclonal Antibodies

A variation in the C-gene derived protein can affect the sequence and/or the conformation of the epitopes recognised by antibodies, which is most relevant in studies with MAbs. In this study the most striking example was observed when using MAb 13D3. In the anti-HBe seropositive patients No. 13 to 15 (Table IV) 50–80% of the hepatocytes were immunoreactive with MAb 13D3 (Fig. 1i), which was comparable to the proportion of positive hepatocytes, detected with the polyclonal antibody (Fig. 1j) and three other MAbs (Table IV). When the same investigation was undertaken with specimens from HBeAg positive patients, the proportion of hepatocytes showing positive immunostaining with 13D3, was markedly reduced or absent (Fig. 1k), when compared with the immunostaining with polyclonal antibody (Fig. 1l). Patients No. 13 to 15 differed from the remaining of Group A not only with respect to their HBeAg/anti-HBe status, but also because they were liver transplant recipients, receiving immunosuppressive treatment. To clarify the difference in the immunostaining pattern with Mab 13D3 we further analysed the recognition of epitope aa 143–146 in liver biopsies from five untreated anti-HBe(+) patients with chronic hepatitis B and 3 HBeAg(+) liver transplant recipients (Table I, Group B). The immunostaining of these liver specimens with MAb 13D3 and polyclonal anti-HBc did not reveal an association with the HBeAg/anti-HBe status or the immunosuppression (Table V). Therefore, the most conceivable explanation for these findings was to assume nucleotide sequence variation that abrogates the recognition of 13D3 epitope. To investigate this possibility, C-gene sequences spanning this epitope were determined in HBV-DNA amplified from serum of all 11 patients shown in Table V. All five patients showing positive 13D3 immunoreactivity, comparable to the polyclonal anti-HBc, were found to be infected with HBV genotype D without any HBc protein sequence variation in the epitope and its flanking region (Fig. 2). In contrast, all other patients were either infected with genotype A (patients 16–18 and 22) which differ from genotype D by a two amino acid insertion, close to 13D3 epitope, or were infected with genotype D with one (patient 23) or several mutations (patient 19) close to the recognition site of MAb 13D3

TABLE V. Detection of Core Protein Epitope aa143–146 in Liver Tissue in Relation to Core Gene Sequence Variation*

Patient no.	OLT	Serum HBeAg/Anti-HBe	Serum HBV-DNA pg/ml	Precore mutations	HBcAg in hepatocytes PAb %	HBcAg in hepatocytes anti-HBc site	HBcAg in hepatocytes Mab %	HBcAg in hepatocytes 13D3 site
13	+	−/+	1845	TAG (28)	80	N/c	80	N/C
14	+	−/+	484	TAG (28)	50	N/c	50	N
15	+	−/+	92	TAG (28)	80	N/c	50	N
16	+	+/−	840	WT	>80	N/C	1	N
17	+	+/−	810	WT	>80	N/C	1	N
18	+	+/−	138	WT	>80	N/C	1	N
19	−	−/+	1200	TAG (28)	30	N/c	0	—
20	−	−/+	420	TAG (28)	50	N/c	30	N
21	−	−/+	380	TAA (28)	50	N/c	50	N/c
22	−	−/+	80	TAA (28)	30	N/c	0	—
23	−	−/+	200	TAG (28)	10	N/c	0	—

*Patient numbers correspond to those in Table I. OLT indicates orthotopic liver transplant recipients with recurrent HBV infection in the graft. WT; Wild type HBV; TAG and TAA indicate a precore stop codon at the position given in parentheses.

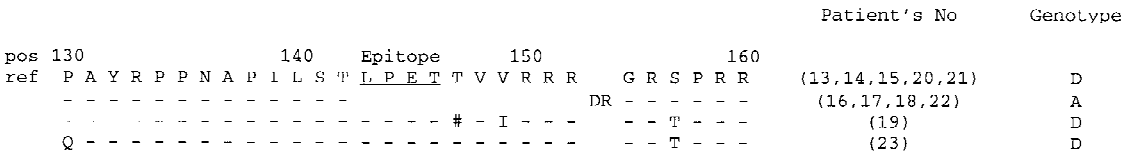

Fig. 2. Carboxyterminal amino acid sequence of the core protein from a reference HBV genome (genotype D, subtype ayw) [Galibert et al., 1979] and of the HBV genomes isolated from various patients. # denotes a heterogeneous population of nucleotide sequences at the given position which may code for S, T, or C.

(Fig. 2). These data indicate that monoclonal antibody 13D3 is able to differentiate between infection of hepatocytes with the wild type of HBV genotype A and genotype D and can sensor for subtle sequence variations occurring even in the vicinity of the recognition site. Similarly, the failure of MAb 11E2 to stain hepatocytes of tissue sections from patients 7 and 8 (Table IV) is most likely due to an amino acid substitution, as revealed by the DNA sequencing. In both patients threonine at codon 12, i.e., two positions downstream of the mapped epitope, was replaced by serine. MAb C1-5 failed to stain the cryostat and paraffin sections of these patients despite the strong positivity with MAb C3-1. The cytoplasmic expression of core protein in these two cases was always stronger than the nuclear with many hepatocytes showing only a cytoplasmic localisation of C-gene products (Fig. 1g). This could be due to the fact that patients 7 and 8 were the only chronic HBV carriers in our study originating from the Far East, they carried identical C-gene nucleotide sequences (region sequenced codon 1 to 150) and showed substantial amino acid sequence variations in reference to genotype A [Valenzuela et al., 1980].

DISCUSSION

Using well defined mouse monoclonal antibodies it was demonstrated that all immunoreactive C-gene derived proteins in HBV infected human hepatocytes are present in the form of assembled core particles. In most patients the largest amount appears to be in the nuclei, as deduced from the intensity of the immunostaining signal. The intensity and the ratio of cytoplasmic to nuclear core expression is not dependent on the ability or inability of the virus to express HBeAg, but on the processing of the liver specimens and the type of antibody used. No evidence was found for intracellular accumulation of non-assembled or denatured core protein or for membraneous localisation of HBeAg.

Naked HBV nucleocapsids have previously been detected by electron microscopy in human hepatocytes. Most of these were found in the nuclei and only occasionally in the cytoplasm [Gudat et al., 1975]. Our results are consistent with this and further indicate that core particles with the same or similar antigenic makeup are present in both cellular compartments. The immunodetection of assembled core particles with MAbs C3-1 and C1-5, which recognize two different conformational epitopes showed variations in the proportion of positive hepatocytes and in the ratio of nuclear/cytoplasmic localization within the same liver specimen, as well as between specimens from different patients. In earlier ultrastructural studies and recently using electron cryomicroscopy combined with image processing, core particles of two different sizes—25 and 30 nm in diameter, have been detected [Cohen and Richmond, 1982; Crowthers et al., 1994]. The populations of the cytoplasmic and nuclear core particles also differ in phosphorylation status [Kann and Gerlich, 1994; Liao and Ou, 1995], which was shown to be associated with a conformational change of the particulate core [Yu and Summers, 1994]. All these factors may have contributed to the differential reactivities observed in this study when using the two monoclonal antibodies, which recognize different conformational epitopes.

The finding that most of the nonenveloped core pro-

tein in hepatocytes of HBV infected patients is in a particulate form was unexpected, in view of some earlier in vitro studies. It was reported that the assembly of core particles is strongly dependent on the concentration of core protein and occurs mainly in the cytoplasm [Zhou et al., 1991, 1992; Seifer et al., 1993]. Our data suggest that the concentration of HBc in the nucleus of infected hepatocytes from chronic HBV carriers is higher than in Xenopus oocyte nuclei and sufficient to drive most of the core protein into the assembled form. If it is true that the viral polymerase, produced in the cytoplasm, acts as a starting point for core particle assembly [Bartenschlager and Schaller, 1993; Kann and Gerlich, 1994], one has to assume either that the core assembly only in the cytoplasm is dependent on the viral polymerase or that substantial amounts of the viral polymerase are transported into the nucleus. Recent data with transfected cells have shown that a substantial fraction of HBV polymerase protein fragments can be present in the nucleus [Schilling and Will, 1996], which is consistent with the latter possibility.

The analysis of specific core epitopes, by sequencing the viral strains and comparing the nucleotide sequence with the immunostaining patterns in liver sections, indicate that immunostaining with MAb can detect C-gene variations. The most striking example was observed with MAb 13D3 which stained sections infected with the wild type HBV genotype D, but not those infected with genotype A. This differential staining is most likely due to the presence of two additional amino acids in genotype A, inserted in the vicinity of the corresponding epitope. The lack of staining of liver tissue from two patients infected with HBV genotype D, which were shown to have mutations close to, but not within the MAb13D3 epitope, corroborates this interpretation. These findings were not expected, as until now the epitope of MAb13D3 was believed to be conformationally independent because it reacted in both peptide ELISA and in immunoblots [Bichko et al., 1993]. The new data suggest that mutations close to the epitope induce a novel conformation not present in the wildtype core protein and therefore abrogate binding of MAb 13D3. Dramatic changes in the immunoreactivity of an epitope, as a result of mutations close to a MAb binding region, may be a more common phenomenon since it was also observed with another MAb—11E2. Thus, a comparative immunostaining with MAb and a polyclonal antibody may be useful in obtaining indirect evidence about HBV C-gene sequence heterogeneity. Moreover, by using double staining of the same liver sections with the antibodies used here it may be possible to study the distribution of a specific mutant strain and the wildtype HBV in individual hepatocytes, tumor and non-tumor tissue, or specific areas of the section. Application of MAbs for immunostaining of liver sections should be particularly useful for the analysis of hepatitis B patients chronically infected with HBV genomes exhibiting deletions and point mutations in the C-gene [Ackrill et al., 1993; Bertoletti et al., 1994; Naoumov et al., 1995, Günther et al., 1995, Marinos et al., 1996]. This may further our understanding of their possible role in HBV pathogenesis and antiviral therapy.

The mechanisms which regulate nuclear core protein localization are not well understood. One report indicated an influence of the cell cycle in cultured cells [Yeh et al., 1993]. This appears to play only a minor role in vivo, as suggested from our results with naturally infected hepatocytes. Even in patients with cirrhosis and severe chronic hepatitis, with hepatocytes in different stages of the cell cycle because of liver cell regeneration, core gene products were predominantly with nuclear localization, similar to the finding in patients with minimal inflammatory reaction in the liver. In a transgenic mouse model the HBV nucleocapsid particles were strictly localized in the nuclei of hepatocytes, except when the nuclear membrane dissolved during cell division and they entered in the cytoplasm [Guidotti et al., 1994]. The cytoplasmic core particles did not re-enter the nucleus and therefore, the presence of cytoplasmic core was found only in recently divided hepatocytes, particularly in regenerating liver of transgenic mice. The hepatic expression of HBcAg in patients with chronic HBV infection is a resultant of both the level of virus replication and the degree of liver inflammation. Earlier studies using polyclonal rabbit anti-HBc on paraffin liver biopsies have suggested that the distribution of HBcAg is associated with liver disease activity with a greater cytoplasmic core expression in patients with chronic active hepatitis in comparison to patients with minimal inflammation [Hsu et al., 1987]. The data in this study revealed that this polyclonal anti-HBc detects predominantly denatured core products and using monoclonal antibodies specific for conformational epitopes we have shown here that there is no strict division between nuclear and nuclear/cytoplasmic core protein localization in relation to liver disease activity, as core particles are usually present in both compartments. Furthermore, the degree of hepatic inflammation and local cytokine production in particular, is an important variable in determining the cellular expression of core protein in hepatocytes. A recent study demonstrated elegantly that interferon-gamma and tumour necrosis factor-alpha, secreted by intrahepatic cytotoxic T lymphocytes, eliminate cytoplasmic HBV nucleocapsids and replicative DNA intermediates without destruction of infected cells [Guidotti et al., 1996]. The unusual pattern of preferential cytoplasmic core staining, observed in patients No. 7 and 8 in this study, is more likely due to the presence of a particular virus population characterized by multiple mutations in the C-gene, which were similar between these two isolates. Mutations possibly inactivating the function of the nuclear localization signals of the core protein [Yeh et al., 1990; Eckhardt et al., 1991], and mutations modulating envelope protein interaction with the core protein could be involved. The influence of preS proteins and of a single mutation in the nuclear localization signal on

the subcellular localisation of core protein is well documented [Yeh et al., 1994a,b, 1995].

Our finding that practically all core protein in hepatocytes of chronic HBV carriers is assembled in core particles is also relevant to the host immune responses. The particulate core protein is more immunogenic than the denatured forms [Schödel et al., 1992]. In athymic mice only the particulate form of HBcAg was able to induce a T cell-independent antibody response [Milich et al., 1986]. We have recently shown that the serum levels of IgM anti-HBc in chronic HBV infection strongly correlate with the degree of hepatocellular damage, both in patients infected with the wildtype HBV or its precore mutant variant [Marinos et al., 1994]. The observation in this study that most of the core protein in hepatocytes is particulate, provides an explanation for this finding, as the release of native core particles from damaged hepatocytes in chronic hepatitis B would activate directly B lymphocytes to secrete IgM antibody to core protein and may also stimulate the function of T helper lymphocytes.

The immunostaining of liver specimens with well defined monoclonal and polyclonal antibodies to HBV nucleocapsid allows fine dissection of the hepatocellular expression of C-gene products in human hepatocytes. In combination with molecular analyses of the viral genomes, this offers a promising approach to study the pathobiology of HBV at a single cell level.

ACKNOWLEDGMENTS

The Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie is supported by the Bundesministerium für Gesundheit und the Freie und Hansestadt Hamburg. The work of H.W. and S.M. was supported by grants from the Wilhelm-Sander-Stiftung and the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie.

We are grateful to Dr. Bernard C. Portmann for the routine histological assessment of liver specimens used in this study. The support from Schering-Plough UK for printing the figures in colour is gratefully acknowledged.

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